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Multi-omic Analyses of Extensively Decayed Pinus contorta Reveal Expression of a Diverse Array of Lignocellulose-Degrading Enzymes.

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1 Multi-omic analyses of extensively decayed *Pinus contorta* reveal expression of diverse array of  
2 lignocellulose degrading enzymes

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20

21 ABSTRACT Fungi play a key role cycling nutrients in forest ecosystems but the mechanisms remain  
22 uncertain. To clarify the enzymatic processes involved in wood decomposition, metatranscriptomics  
23 and metaproteomics of extensively decayed lodgepole pine were examined by RNAseq and LC-

MS/MS, respectively. Following *de novo* metatranscriptome assembly, 52,011 contigs were searched for functional domains and homology to database entries. Contigs similar to basidiomycete transcripts dominated and many of these were most closely related to ligninolytic white rot fungi or cellulolytic brown rot fungi. A diverse array of carbohydrate active enzymes (CAzymes) representing a total of 132 families or subfamilies were identified. Among these were 672 glycoside hydrolases including highly expressed cellulases or hemicellulases. The CAzymes also included 162 genes encoding redox enzymes classified within Auxiliary Activity (AA) families. Eighteen of these were manganese peroxidases, key components of ligninolytic white rot fungi. Expression of other redox enzymes supported the working of hydroquinone reduction cycles capable of generating reactive hydroxyl radical. The latter has been implicated as a diffusible oxidant responsible for cellulose depolymerization by brown rot fungi. Thus, enzyme diversity and the coexistence of brown and white rot fungi suggest complex interactions of fungal species and degradative strategies during the decay of lodgepole pine.

**IMPORTANCE** The deconstruction of recalcitrant woody substrates is a central component of carbon cycling and forest health. Laboratory investigations have contributed substantially toward understanding mechanisms employed by model wood decay fungi, but few studies have examined the physiological processes in natural environments. Herein, we identify the functional genes present in field samples of extensively decayed lodgepole pine (*Pinus contorta*), a major species distributed throughout the North American Rocky Mountains. The classified transcripts and proteins revealed a diverse array of oxidative and hydrolytic enzymes involved in the degradation of lignocellulose. The evidence also strongly supports simultaneous attack by fungal species employing different enzymatic strategies.

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50 Fungi play a critical role in recycling of forest carbon via wood decomposition. Significant effort has  
51 focused on describing the microbial communities using conventional plating techniques, observations  
52 of conspicuous fungal fruiting bodies (1), enzyme assays (2) and metagenomics analyses of rRNA and  
53 other conserved genes (3-5). The complexity and interactions among physiologically active wood  
54 decay microbes remain poorly understood, although recent work on forest soils and litter may be  
55 relevant, at least in terms of experimental tools (6-11).

56 Two forms of wood decay are generally recognized (12, 13). Brown rot fungi rapidly depolymerize  
57 cellulose via oxidative systems. Bulk lignin is left behind as a polymeric residue. In contrast, white rot  
58 fungi degrade and metabolize lignin while cellulose is hydrolyzed by conventional Carbohydrate  
59 Active Enzymes (CAzymes) (14). Except for rare cases (15), genome annotation supports these  
60 classifications; white rot fungi feature genes encoding ligninolytic peroxidases and numerous  
61 cellulases while brown rot fungi have few, if any, of these genes (16). Efficient degradation and  
62 mineralization of native lignin are accomplished almost exclusively by white rot basidiomycetes, some  
63 of which are closely related to brown rot fungi. The efficiency by which these fungi degrade cellulose  
64 and lignin has generated considerable interest in bioprocess development.

65 Toward the identification of enzymes directly involved in the natural decomposition of wood,  
66 metatranscriptome and metaproteome investigations were performed on decayed lodgepole pine (*Pinus*  
67 *contorta*), a fire-adapted species widely distributed throughout the North American Rocky Mountains.

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## 70    **RESULTS**

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72        **Metatranscriptome.** Analyses were focused on Poly(A) RNA purified from extensively decayed  
73 lodgepole pine samples collected in western Montana national forests. To provide a broad perspective,  
74 sampling included distinct locations and fire histories. Avoiding direct contact with soil/litter, the loose  
75 material was collected from the logs' upper surface. A total of  $2.9 \times 10^8$  Illumina-RNA-Seq reads were  
76 generated from the amplified poly(A) RNA. De novo assembly yielded 274,233 contigs, each with a  
77 minimum of 100 reads. To add confidence to BLASTx queries, these were further filtered to 52,011  
78 contigs over 750 bp in length (Table 1).

79        BLASTx searches of metatranscriptome contigs against NCBI RefSeq, GO, InterPro and EC  
80 supported putative classifications and revealed conserved protein domains directly supporting function  
81 and/or features potentially involved (e.g. secretion signals, transmembrane helices) (Table 1). Using a  
82 conservative e-value threshold ( $10^{-15}$ ), 41% of the contigs were related only to hypothetical proteins or  
83 otherwise unassigned (Figure 1). A total of 1,316 putative CAzyme-encoding transcripts were  
84 identified. Impressive numbers of transporters (1,631) and cytochrome P450s (203) were also found  
85 and, although their precise function is difficult to predict, some are likely to play an important role in  
86 the uptake and metabolism of low molecular weight products during cell wall degradation. A  
87 substantial number of extracellular proteases together with oligopeptide and amino acid transporters  
88 (Dataset S1) are probably indicative of nitrogen scavenging within the N-limited substrate. Not  
89 surprisingly, approximately 50% of the Refseq hits were encoding intracellular structural proteins (e.g.  
90 actin) or enzymes involved in protein synthesis (e.g. ribosomal proteins) and central metabolism (e.g.  
91 Glyceraldehyde-3-phosphate dehydrogenase). Without doubt, relaxing blast thresholds and/or

92 inclusion of contigs < 750 bp would identify additional sequences, possibly including those encoding  
93 small secreted proteins (17). A detailed description of contigs is presented in supplemental Dataset S1.

94 Of the 1,316 CAzymes, 162 were classified as members of an Auxiliary Activities (AA) family  
95 (Figure 2; (18). Clearly demonstrating white rot ligninolysis, eight contigs encoding manganese-  
96 dependent lignin peroxidases (MnPs) were among the 30 most highly expressed AA-encoding genes  
97 (Table 2). Abundant transcripts corresponding to eight alcohol oxidases were also identified, and all  
98 were closely related to *Gloeophyllum trabeum* methanol oxidase (19). Two benzoquinone reductases  
99 may participate in a redox cycle tied to hydroxyl radical generation during brown rot decay (20-22).  
100 Surprisingly, the highest transcript levels among AA-encoding genes were attributed to lytic  
101 polysaccharide monooxygenases (LPMO; Table 2). Previously classified as GH61s, these copper  
102 dependent enzymes have been shown to cleave C1 or C4 glycosidic bonds [reviewed in Ref. (23)].

103 Beyond the AA families, 1,154 additional CAzymes were identified, including 672 glycoside  
104 hydrolases (GHs), 154 glycosyl transferases (GTs), 105 carbohydrate esterases (CEs), 13  
105 polysaccharide lyases (PLs) and 210 carbohydrate binding modules. Glycoside hydrolase families  
106 associated with cellulose degradation, e.g. GH5\_5, GH6, GH7, GH12, GH45, were particularly  
107 abundant (Figure 3; Table 3). Total transcripts classified as the ‘exo’-acting cellobiohydrolases (GH7s  
108 and GH6s) were substantially increased due to the activity of relatively few, but highly expressed  
109 genes (Figure 3). Similarly, of 25 GH5\_5 contigs, 4921502\_1 and 1142146\_1 (Table 3)  
110 disproportionately contributed 45% of the total reads (Figure 3; Dataset S1). Families GH7, GH6 and  
111 GH5\_5 accounted for 25%, 15% and 15% of the cellulase reads, respectively.

112 Enzymes broadly characterized as hemicellulases were among the most highly expressed  
113 transcripts and included GH114 Endo  $\alpha$ -1-4-polygalactosaminidase (3859657\_1), GH10 endo- 1,4- $\beta$   
114 xylanase (3561636\_2), GH53 endo-1,4- $\beta$ -galactanase and a GH27  $\alpha$ -galactosidase (910111\_1) (Table

115 3). The numbers of contigs and total reads assigned to potential hemicellulase families are shown on  
116 Figure 4. Several of these are involved in the degradation of galactoglucomannan,  
117 arabinoglucuronoxylan and arabinogalactan, all of which are components of softwood hemicellulose.  
118 The complete breakdown of these substrates may involve the combined activities of multiple GH  
119 families. For example,  $\beta$ -mannanases belonging to GH2, GH5\_7 and GH5-41 represented 15% of the  
120 hemicellulose-assigned reads (Dataset S1). In addition to plant cell wall degradation,  $\beta$ -1,3-glucans and  
121 chitin from insects and fungal cell walls are likely substrates for GHs such as GH18 chitinases. Beyond  
122 these hydrolases, CEs associated with the removal of acetyl substitution from hemicellulose and chitin  
123 (CE1, CE4 and CE16) were major components of the transcriptome (Dataset S1).

124 Contigs with putative functions were most frequently related to basidiomycetes (Figure 5, 3S;  
125 Dataset S1), although sequences closely related to other eukaryotes, or their viruses, were also  
126 detected. Of the 52,011 contigs, 39,765 gave significant BLASTx scores e-values ( $<10^{15}$ ) to the NCBI  
127 Refseq database. Translations of these contigs, plus the extended ORFs of those contigs without  
128 significant BLASTx scores, were queried against the NCBI non-redundant (NR) protein database. A  
129 total of 39,973 proteins gave significant hits (e-values  $<10^{15}$ ), and these were distributed among 2,810  
130 species including 1,387 species with a single hit. Forty-two species accounted for approximately 50%  
131 of the top BLASTp hits (Figure 5, Panel A). This overall pattern of similarity to basidiomycete genes  
132 was also observed for a much smaller subset of 1,316 CAZy-encoding genes (Figure 5 panel B). The  
133 skewed representation toward basidiomycetes is also evident when considering up to ten database  
134 entries most closely related to each contig (Fig. 3S).

135 Many genes involved in lignocellulose deconstruction were highly expressed including the  
136 abovementioned exo- and endo-acting cellulases, transporters and oxidoreductases (Tables 2, 3 and  
137 S1). In some instances, possible prokaryote sequences were detected. For example, the predicted

138 amino acid sequence of 1384861\_1, a putative cellobiohydrolase (Table 3), was 43% (e-value  $10^{-102}$ )  
139 identical to *Sorangium cellulosum* (KYG02679). However, the contig sequence is also similar to  
140 various fungal accessions such as OAL51409 from *Pyrenochaeta sp.* but polyadenylation is absent, as  
141 expected if originating from prokaryotic organisms. Evidence supporting the prokaryotic origins of  
142 other highly expressed genes is more compelling. Specifically, contigs 94066\_1, 4739131\_4 and  
143 2374420\_1 are among the 30 most highly expressed glycoside hydrolases (Table 3) and most closely  
144 related to *Kutzneria sp* WP\_084578858), *Enhygromyxa salina* (KIG15205) and *Actinobacteria*  
145 *bacteria* (OLB81315) genes, respectively (Dataset S1). All have short 3' poly(A) tracts of 16 to 24  
146 residues and, in each case, the 10 most closely related database entries are also derived from bacterial.

147 A total of 28 contigs were most closely related to cDNAs derived from an “uncultured eukaryote”  
148 in *Picea abies* forest soils (7). Twenty-two of these encode putative CAZy domains (Dataset S1). The  
149 deduced amino acid sequence of a highly expressed GH45-encoding gene (4034495\_1; Table 3) is  
150 76% identical to accession CCA94939 (7).

151 **Metaproteome.** MS/MS data matched 1,964 proteins corresponding to 1,935 contigs. Of these  
152 mass spectrometry-identified proteins, the majority were encoded by orthologous sets of housekeeping  
153 genes such as 40S and 60S ribosomal proteins, actin and glyceraldehyde-3-phosphate dehydrogenase  
154 (Dataset S1). Sixty-seven proteins corresponded to hypothetical proteins, 42 were predicted proteins  
155 and 62 were not assigned by NR BLASTp. On the other hand, 24 proteins were classified by Auxiliary  
156 Activities, and four Dye Decolorizing Peroxidases (DyP; Reviewed in Ref. (24) ) were also identified  
157 (Table 4). Other CAzymes included 11 carbohydrate esterases and 23 glycoside hydrolases categorized  
158 as cellulases and hemicellulases (Table 5). The semi-quantitative measures of protein abundance were  
159 not correlated with the number of reads, a common observation often related to solubility and cellular  
160 targeting (intracellular, extracellular, membrane bound), protein stability, substrate binding, molecular

weight and/or access to trypsin cleavage sites. Previous studies have shown high numbers of basidiomycetous proteins in forest litter, an observation attributed to relatively high C/N ratios (11, 25).

Enzymatic activities of cellobiohydrolase,  $\beta$ -1,4 glucosidase,  $\alpha$ -1,4-glucosidase,  $\beta$ -1,4-N-acetylglucosaminidase,  $\beta$ -1,4-xylosidase,  $\beta$ -glucuronidase, L-leucine aminopeptidase and peroxidase activity were detected at the three sampling points (Fig. S3). Because no laccase transcripts or proteins could be clearly identified, polyphenol oxidase activity may be due to non-specific oxidations of the L-DOPA substrate (Table S4). However, other multicopper oxidases, including ferroxidase-like enzymes (5217211\_1, 2197006\_3, 1400590\_1; Dataset S1), may have contributed to the L-DOPA activity.

## DISCUSSION

Filamentous fungi play a key role in depolymerizing, degrading and mineralizing the major components of woody cell walls, including cellulose, hemicellulose and the recalcitrant lignin. Only a fraction of the species has been isolated in pure culture. Moreover, the laboratory conditions employed with model white rot and brown rot fungi fail to mimic natural decay processes. To identify key enzymes and further understand lignocellulose deconstruction we have examined the metatranscriptome and metaproteome of extensively decayed lodgepole pine. In aggregate, the results support complex enzymatic interactions carried out by diverse microbial communities.

The majority of the classified sequences were most closely related to Basidiomycota, followed by cellulolytic Ascomycota, slime molds and other Protista. Species-level taxonomic assignments are likely biased by the skewed representation of genomes available in the NCBI databases. This is particularly problematic when discriminating brown rot and white rot fungi (15), unless certain white

184 rot-specific genes are detected such as the MnPs (Table 2, S1). In aggregate, our results strongly  
185 suggest that both white and brown rot fungi are simultaneously active in decaying lodgepole pine  
186 (Fig.5 and 3S). Our analysis cannot resolve fine spatial relationships between fungi and their  
187 interactions (e.g. mutualistic, antagonistic, neutral) remain uncertain. However, the findings are  
188 consistent with the coexistence of white and brown rot fungi following laboratory inoculation of spruce  
189 logs and placement in forest settings (5, 26, 27). More recently, ITS2 analyses of Norway spruce  
190 (*Picea abies*) logs in unmanaged stands identified various basidiomycetes, including several white and  
191 brown rot species (28). Mäkipää et al reported that most species were rare and that the top 30  
192 accounted for 50% of all OTUs (28). Our BLASTp counts suggest similar levels of species richness,  
193 with the top 30 hits accounting for 43% (18,952) of the contigs. However, we did not observe abundant  
194 mycorrhizal species (28), and our results surely underestimate species diversity because of the  
195 dependency on physiological activity as well as the lability of RNA targets. These results stand in  
196 contrast to metatranscriptome investigations of *Pinus taeda* where few mycorrhizal species are active  
197 (29). In this context, we recognize that the observed patterns would be quite different on other wood  
198 species during wet and/or cool conditions. Our sampling was confined to *P. contorta*, during a dry  
199 period in early summer and, although sampling was not designed to invite statistically valid  
200 comparisons, there seemed relatively little difference in the fire-disturbed versus undisturbed samples  
201 (Table S2; Fig. S1 and S2).

202 Unexpectedly, the second most common top BLAST hits were *Phialocephala* spp. (Fig. 5 and 2S).  
203 A well-established endophyte of conifer needles, *P. scopiformis* produces the anti-insectan compound  
204 rugulosin and confers increased tolerance to spruce budworm. The root endophyte *Phialocephala*  
205 *subalpina* (30, 31) is closely related to *P. scopiformis* and, together, these species constitute 5.8%  
206 (2,542) of the total number of top hits. Possibly, an undescribed *Phialocephala* sp or variant strain

207 encode the observed transcripts and peptides. The genomes of *P. scopiformis* (32) and *P. subalpina*  
208 feature numerous CAzymes and, in view of the expression patterns reported here, it seems probable  
209 that filamentous basidiomycetes and ascomycetous *Phialocephala spp.* participate in the late stages of  
210 *P. contorta* decay.

211 Our analyses identified an array of genes directly and indirectly involved in lignocellulose  
212 degradation. Eighteen MnP-encoding contigs were present (Dataset S1), and peptides matching eight  
213 of these were detected (Dataset S1). Generally considered ligninolytic enzymes, amino acid alignments  
214 were 53% to 67% identical to *Phanerochaete chrysosporium* MnP1 (accession Q02567) and, within  
215 aligned regions, key residues involved in Mn binding and catalysis were conserved (33). In contrast to  
216 MnPs, which are widely distributed among white rot fungi (12, 34), lignin peroxidases and versatile  
217 peroxidases were not detected. We also observed an array of alcohol oxidases (AA3\_3s) that may play  
218 a role in supplying extracellular H<sub>2</sub>O<sub>2</sub> to the peroxidases. The role of six DyP peroxidases, three heme-  
219 thiolate peroxidases (HTPs) and six chloroperoxidases in ligninolysis, if any, remains unclear (35).  
220 Transcripts and activities of these enzymes have been reported in forest litter (36) and, although  
221 lacking the oxidative potential to attack lignin, they may be involved in the oxidation and  
222 hydroxylation of aromatic metabolites derived from lignin (35). The peptides corresponding to four  
223 Dyp genes were identified, but none detected for the chloroperoxidases and HTPs. Neither transcripts  
224 or peptides were identified for laccases suggesting no significant role in ligninolysis, at least in the late  
225 stages of wood decay.

226 Other redox enzymes assigned to AA families provide additional insight into degradative  
227 mechanisms. We were unable to detect LPMO peptides by MS/MS, but the genes (AA9) were  
228 particularly numerous and the transcripts abundant with 65 contigs and 93,889 transcripts (Table 2;  
229 Figure 2; Dataset S1). Assuming these Cu-dependent enzymes are secreted, they likely play an

230 important role in cellulose cleavage, especially the 12 genes that feature a CBM1 domain (Dataset S1).  
231 They may also oxidize xyloglucan and glucomannans as recently demonstrated for a LPMO from the  
232 brown rot fungus *Gloeophyllum trabeum* (37). Interestingly, LPMO activity was recently shown  
233 dependent on H<sub>2</sub>O<sub>2</sub> (38). This may partly explain the number (26), high transcript levels (60,929 reads)  
234 and Peptide Spectrum Matches (PSMs) of putative alcohol oxidases (Table 2, 5 and S1). These family  
235 AA3 enzymes generate peroxide from various substrates, including methanol. Both white rot and  
236 brown rot fungi demethylate lignin and, in the case of *Gloeophyllum trabeum* brown rot, evidence  
237 suggests that methanol oxidase contributes peroxide to advance the Fenton reaction (19).

238 The mechanism(s) of brown rot decay remain unsettled. Recent genome annotations make clear  
239 that cellobiose dehydrogenase is not essential, as this multifunctional enzyme is absent from the  
240 genome of several efficient brown rot fungi. Our analysis also failed to identify the transcript in  
241 decayed lodgepole pine. On the other hand, the data is consistent with hydroxyl radicals generated via  
242 benzoquinone reductase and oxalate-Fe<sup>3+</sup> chelates (20-22). Specifically, we identified 25 putative  
243 benzoquinone reductases (32,831 reads), 20 oxalate decarboxylases (28,450 reads) and an oxalate  
244 transporter which, together, may modulate hydroxyl radical production (39)(Dataset S1). In contrast,  
245 our inability to detect any laccase transcripts undermines its involvement in the process (40-42).

246 Without doubt, lodgepole pine decay involves the combined activities of functionally diverse  
247 enzymes and fungal species. The synergistic activities of conventional endo- and  
248 exocellobiohydrolases are well known. More recently, LPMO has been shown to boost the  
249 performance of these hydrolases (43, 44). Although our study selected for eukaryotic mRNA, genes  
250 most closely related to bacteria were occasionally identified. Several studies have implicated bacteria  
251 as part of complex consortia involved in wood decay (45-48); reviewed in Ref. (49). We also found  
252 genes most closely related to slime molds and other protists as well as several bacteria many of which



253 have been associated with decomposition. The enzymatic machinery and interactions among these  
254 species merit further investigation.

255

## 256 MATERIALS AND METHODS

257 **Sample description and RNA preparation.** Loose and crumbling material from the upper  
258 surface of three extensively decayed lodgepole pine logs were collected in western Montana July,  
259 2014. The logs were in direct contact with the ground. No clear zones of fungal colonization or fruiting  
260 bodies were observed on the decayed wood or in the immediate vicinity. However, portions of the  
261 decayed surfaces had dark cubicle regions interspersed with lighter colored fibrous areas, often  
262 associated with brown rot and white rot, respectively (Figure S5). Locations included U.S. Forest  
263 Service's Tenderfoot Creek Experimental Forest (TCEF) at 46.923333, -110.869444 (N 46 55' 24" W  
264 110 52" 10) (Altitude 2238 m). This specific site was within a mature lodgepole pine stand with a  
265 dense canopy, sparse ground cover and no history of fire in over 140 years. Two samples were  
266 collected near Gibbon's Pass, on the Bitterroot National Forest, MT. The area was the site of a  
267 catastrophic fire in 2001 and, at the time of collection, featured a dense stand of lodgepole saplings.  
268 Considerable ground cover was predominantly *Xerophyllum tenax*, *Lupinus sericeus* and *Vaccinium*  
269 *scoparium*. The GP1 log was in a relatively flat area at 45.754444, -113.909444 (N 45 45' 16" W  
270 113.54' 34) (altitude 2146 m), whereas the GP2 log was located on an incline at 45.755278, -  
271 113.910556 (N 45 45' 19" W 113.54' 38) (altitude 2138 m). The length of time during which the trees  
272 were down could not be determined, although at the time of fall, cellulose, hemicellulose and lignin are  
273 estimated to be 44%, 31%, and 27%, respectively (50). All samples were ground with an electric blade  
274 coffee grinder, passed through a 2 mm sieve, shipped overnight on ice and stored at -80C.

275        Approximately 1 gram of the ground samples were extracted with the MO BIO RNA Isolation Kit  
276 (Carlsbad, CA; catalog # 12866-25) according to the manufacturer's recommendations. The final total  
277 RNA pellet was suspended in 100 ul water. Oligo (dT)<sub>25</sub> Dynabeads (Oslo, Norway) was prepared as  
278 suggested by the manufacturer. Seventy microliters of the beads were washed and suspended in 100 µL  
279 of binding buffer (20mM Tris-HCl, pH 7.5, 1.0M LiCl, 2mM EDTA). To eliminate secondary  
280 structure, RNA was incubated at 65°C for 2 minutes and immediately placed on ice. The RNA and  
281 bead solution were mixed and, after occasional inverting over a 5 min period, placed in a Dynal  
282 magnet stand. The beads were washed and re-captured twice and the Poly(A) RNA eluted with 10 ul  
283 water. The Poly(A) RNA was incubated at 70°C for 10 minutes and amplified following the  
284 MessageAmp II protocol (Invitrogen, ThermoFisher Scientific). The aRNA quantity and quality were  
285 assessed by a Qubit fluorometer (ThermoFisher Scientific) and a 2100 BioAnalyzer (Agilent),  
286 respectively.

287        RNA-Seq libraries were prepared and sequenced by the U.S. Department of Energy Joint Genome  
288 Institute (Walnut Creek, CA). mRNA was fragmented and reversed transcribed using random  
289 hexamers and SSII (Invitrogen) followed by second strand synthesis. The fragmented cDNA was  
290 treated with end-pair, A-tailing, adapter ligation, and eight cycles of PCR. qPCR was used to  
291 determine the concentration of the libraries. The prepared libraries were quantified using KAPA  
292 Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-  
293 time PCR instrument. The quantified libraries were then prepared for sequencing on the Illumina  
294 HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v4, and Illumina's cBot  
295 instrument to generate a clustered flowcell for sequencing. Sequencing of the flowcell was performed  
296 on the Illumina HiSeq-1TB sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 2x150  
297 indexed run recipe. Fastq files were deposited in the Sequence Reads Archive under biosample

298 accessions SAMN07573382 (GP1), SAMN07573383 (TCEF) and SAMN07573384 (GP2). The three  
299 sample files were combined, without normalization, for final assembly. DNASTAR's SeqMan NGen  
300 (v 14.1) was used to perform *de novo* transcriptome assemblies with default parameters (mer size:21;  
301 clustering match: 97%) with a minimum of 101 sequences per contig (average: 590). The resulting  
302 assemblies were sorted according to the number of reads and length with SeqMan Pro. Those contigs  
303 exceeding 750 bp were initially BLASTx queried against the Refseq protein database and EMBL-EBI  
304 was used to predict Interpro domains as well as transmembrane helices, secretion signals and enzyme  
305 codes. The top BLASTx hits were used to guide protein translations of 39,765 genes and, for the 12,246  
306 contigs lacking significant similarity, the longest ORFs among six strands were translated. For  
307 BLASTp searches of NR, contigs were translated based on BLASTx results or, in the case of little or  
308 no significant sequence homology, the longest ORFs were used. Transeq 5.0.0 was used to translate  
309 ORFs within all contigs in all frames for the mass spectrometry database (below) (51)(52). Gene  
310 Ontology annotations were via blast2Go.

311 **Protein extraction and mass spectrometry identification.** Total soluble protein was extracted  
312 from approximately 5 g Wiley ground wood samples using the NoviPure Soil Protein Extraction kit  
313 (MO BIO, Qiagen Carlsbad, CA, catalogue # 30000-20). NanoLC-MS/MS was used to identify  
314 proteins in as described (53-55).

315 Crude protein extracts were further purified with TCA precipitation followed by multiple acetone  
316 washes. The pellets were resolubilized to [~1mg/ml] of dry weight:liquid with 8M Urea and aliquots  
317 taken for protein concentration determination (PIERCE™ 660nm Protein Assay kit, ThermoFisher  
318 Scientific). 25µg of urea solubilized protein extract was 10x concentrated with liquid/liquid extraction  
319 (methanol:chloroform:water), trypsin/LysC digested, OMIX C18 SPE cleaned up (Agilent  
320 Technologies) and finally 1.5µg loaded for nanoLC-MS/MS analysis using an Agilent 1100 nanoflow

321 system (Agilent Technologies) connected to a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-  
322 Orbitrap Elite™, ThermoFisher Scientific) equipped with an EASY-Spray™ electrospray source.  
323 Chromatography of peptides prior to mass spectral analysis was accomplished using capillary emitter  
324 column (PepMap® C18, 3μM, 100Å, 150x0.075mm, Thermo Fisher Scientific) onto which 2μl of  
325 purified peptides was automatically loaded. NanoHPLC system delivered solvents A: 0.1% (v/v)  
326 formic acid , and B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid at 0.50 μL/min to load the  
327 peptides (over a 30 minute period) and 0.3μl/min to elute peptides directly into the nano-electrospray  
328 with gradual gradient from 3% (v/v) B to 20% (v/v) B over 154 minutes and concluded with 12  
329 minute fast gradient from 20% (v/v) B to 50% (v/v) B at which time a 5 minute flash-out from 50-95%  
330 (v/v) B took place. As peptides eluted from the HPLC-column/electrospray source survey MS scans  
331 were acquired in the Orbitrap with a resolution of 120,000 followed by MS/MS fragmentation of 20  
332 most intense peptides detected in the precursor MS1 scan from 380 to 1800 m/z; redundancy was  
333 limited by dynamic exclusion. Raw data files were imported into Thermo Proteome Discoverer (PD  
334 version 2.2.0.388) for database searching and assignment of identification significance. A user-defined  
335 metagenomic database was compiled from 6 frame translations of 52,011 assembled contigs (>750bp)  
336 generating 312,066 protein entries. Sequest HT search engine (within PD) assigned peptide and protein  
337 identifications within 1% False Discovery Rate using 15 ppm mass tolerance for peptide and 0.6 Da  
338 for a fragment ion mass. Variable methionine oxidation with asparagine and glutamine deamidation  
339 plus fixed cysteine carbamidomethylation was specified. Further stringency for an acceptable  
340 identification was applied by requiring at least two peptides per individual protein and Xcorr value of  
341 1.8 or greater per individual peptide spectrum match. The raw mass spectrometry data was deposited to  
342 Chorus repository (chorusproject.org, ID#1478) for public sharing and visualization.

343 It should be noted that under the conditions employed, mass spectrometry detection of extracellular  
344 proteins is subject to false negative results due to the frequency of trypsin cleavage, low molecular  
345 weight, substrate binding and/or proteolytic degradation. False positive results in contrast, are unlikely.

346 The potential activity of 10 extracellular wood-degrading enzymes was quantified on a limited  
347 amount of ground sample (<40 mg dry weight) from each location: cellobiohydrolase (CBH, an  
348 exocellulase),  $\beta$ -glucosidase (BG, which hydrolyzes cellobiose into glucose),  $\alpha$ -glucosidase (AG,  
349 which hydrolyzes starch),  $\beta$ -xylosidase (BX, which degrades the xylose component of hemicellulose),  
350  $\beta$ -glucuronidase (BGLU, which degrades the beta-glucuronic acid component of hemicellulose),  $\beta$ -N-  
351 acetyl-glucosaminidase (NAG, which breaks down chitin), acid phosphatase (AP, which releases  
352 inorganic phosphate from organic matter), leucine-aminopeptidase (LAP, which breaks down  
353 polypeptides), polyphenol oxidase (PPO, which oxidizes phenols), and peroxidase (PER, including  
354 oxidases that degrade lignin). Samples were stored frozen at  $-80^{\circ}\text{C}$  after collection and analyzed as  
355 described (56), with the following modification: to ensure detectable activity, the LAP assay was  
356 conducted in 0.2M phosphate buffer, pH 6.8, with 1 mM fluorogenic substrate. All enzyme activities  
357 were calculated as nmol activity per g per hr and ln-transformed prior to interpretation.

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369

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Table 1. Metatranscriptome and Metaproteome  
summary

RNA-seq reads	291,596,160
Contigs > 100 reads	274,233
Average length, bp	590
Contigs >100 reads; >750bp	52,011
IPR domains	42,307
GO assignments	33,523
GO after merging	116,997
Confirmed IPS GOs	103,837
EC assignments	7,901
Oxidoreductases	2,083
Transferases	1,553
Hydrolases	3,242
Lyases	556
Isomerases	389
Ligases	281
TMHs predicted	10,510
Eukaryotic secretion signal	2,705
Protein detected <sup>1</sup>	1,935

<sup>1</sup>Mass spectroscopy detected proteins using 6-frame translated contigs.

Table 2. Contigs predicted to encode proteins with Auxiliary Activities (AA) ordered by sequences reads.<sup>1</sup>

Contig	Reads	Length	Family <sup>2</sup>	Putative function	e-Value <sup>3</sup>	Sim mean, % <sup>4</sup>	Comment <sup>5</sup>
5367253_3	10056	1225	AA3_3	Methanol oxidase	0	93.1	MS
2929739_1	9675	873	AA9	LPMO	3.35E-54	57.9	SS
1507360_1	9562	1007	AA6	Benzoquinone reductase	5.87E-86	75.1	MS
4793784_4	8517	892	AA2	Manganese peroxidase	7.12E-98	73.5	
3105967_1	8470	824	AA9	LPMO	6.42E-95	70.8	SS
1303515_1	7776	1316	AA2	Manganese peroxidase	1.91E-158	76	
798557_1	7736	1563	AA3_3	Alcohol oxidase	0	90.8	MS
4253668_1	7400	862	AA9	LPMO	4.27E-113	76.1	SS
1203164_1	6279	820	AA9	LPMO	1.78E-72	84.1	CBM1
694467_2	5891	1568	AA9	LPMO	5.19E-68	72.2	
3968694_1	5536	1140	AA2	Manganese peroxidase	2.44E-114	77.9	
3834150_1	5521	862	AA6	Benzoquinone reductase	5.01E-103	82.9	MS
3968694_2	5211	1057	AA2	Manganese peroxidase	1.94E-80	76.4	
3406352_1	4719	1151	AA2	Manganese peroxidase	1.00E-128	75	
536886_1	4574	1455	AA3_3	Alcohol oxidase	0	93.8	MS
5221162_1	4332	865	AA9	LPMO	4.28E-36	65.7	
4793784_3	4023	932	AA2	Manganese peroxidase	7.40E-106	72.2	MS
1201987_1	3747	1074	AA9	LPMO	5.11E-116	83.2	SS
2929739_3	3640	915	AA9	LPMO	1.91E-47	60.4	SS; MS
2280897_3	3374	1119	AA3_3	Alcohol oxidase	0	92.8	MS
4793784_1	3310	901	AA2	Manganese peroxidase	3.24E-92	71.3	MS
4582340_1	3263	929	AA9	LPMO	2.36E-83	82	CBM1
2280897_7	3087	1095	AA3_3	Alcohol oxidase	8.37E-148	92	MS
798557_5	3018	1095	AA3_3	Alcohol oxidase	0	87.6	MS
1303515_3	2984	1055	AA2	Manganese peroxidase	1.35E-119	80.1	
2353562_1	2843	1263	AA3	GMC oxidoreductase	3.97E-59	50.4	
3751681_1	2838	912	AA9	LPMO	2.47E-100	79.7	SS
798557_17	2693	1051	AA3_3	Alcohol oxidase	9.20E-171	89	MS
4109481_1	2461	1271	AA3_2	GMC oxidoreductase	1.38E-111	65.3	



1227884_4	2455	1033	AA3_3	Alcohol oxidase	1.06E-175	91	MS
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377 <sup>1</sup>Complete information, including BLASTx analyses, Interpro and Gene Ontology designations, and  
 378 mass spectrometry results are listed in supplemental Dataset S1.

379 <sup>2</sup>CAZy families identified by dbCAN (Yin et al 2012 NAR doi: [10.1093/nar/gks479](https://doi.org/10.1093/nar/gks479))

380 <sup>3</sup>e-value of top BLASTx hit.

381 <sup>4</sup>Average sequence similarity to the top ten BLASTx hits.

382 <sup>5</sup>SS, SignalP determined secretion signal; MS, LC-MS/MS identified proteins; CBM1, Carbohydrate  
 383 Binding Module family 1.

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385

Table 3. Contigs predicted to encode glycoside hydrolases (GH) ordered by sequences reads<sup>1</sup>

Contig	Reads	Length	Family <sup>2</sup>	Putative function	e-Value <sup>3</sup>	sim mean, % <sup>4</sup>	comment <sup>5</sup>
3240852_1	42911	1551	GH7	Cellobiohydrolase 1	0	78.6	SS; MS
6091156_1	25158	1281	GH6	Cellobiohydrolase 2	2.21E-98	59.3	SS; MS
4924579_1	24747	1587	GH13	1,4- $\alpha$ -glucan branching enzyme	0	88.8	
94066_1	24422	1169	GH5	Endo- $\beta$ -1,4-glucanase	1.91E-134	69.5	SS
4034495_1	23449	1472	GH45	Endo- $\beta$ -1,4-glucanase	2.51E-32	56.9	SS
4921502_1	19419	1252	GH5_5	Endo- $\beta$ -1,4-glucanase	8.65E-160	83	SS; MS
5743057_1	18678	845	GH12	Endo- $\beta$ 1,4-glucanase	2.54E-115	77.9	SS; MS
4739131_4	17714	1657	GH5	Glycoside hydrolase	2.11E-74	54.9	SS
1384861_1	16216	1295	GH6	Cellobiohydrolase 2	1.57E-88	58.1	SS; MS
6836211_1	15440	1335	GH18	Chitinase	1.49E-62	64.2	
				Endo- $\alpha$ -1,4			
3859657_1	14542	1457	GH114	polygalactosaminidase	8.40E-90	69.5	
3529359_1	14380	1570	GH16	$\beta$ -1,3-glucanase	1.72E-109	68.5	SS
3589366_1	13195	1464	GH7	Cellobiohydrolase 1	0	78.5	SS; MS
3098980_1	13193	1072	GH16	Endo-1,3- $\beta$ -glucanase	2.52E-106	70.4	
2908735_1	10927	1113	GH79	Glycoside hydrolase	1.82E-74	68.1	
3561636_2	9572	1222	GH10	Endo-1,4- $\beta$ xylanase	9.07E-158	69.7	SS
65078_1	9266	1125	GH7	Cellobiohydrolase 1	1.99E-144	75.9	CBM1
5394650_1	8876	1568	GH5_22	Glycoside hydrolase	1.36E-81	66	
1142146_1	8843	1125	GH5_5	Endo- $\beta$ -1,4-glucanase	1.97E-151	76.8	
4728183_1	8453	1939	GH128	Glycoside hydrolase	1.89E-125	70.9	SS
98832_1	7998	943	GH128	Glycoside hydrolase	5.62E-88	65.9	
2374420_1	7848	1075	GH9	Glycoside hydrolase	3.18E-50	52.2	
2989759_1	7548	1266	GH128	Glycoside hydrolase	1.48E-50	52.2	
247968_1	7473	1442	GH5_40	Glycoside hydrolase	2.96E-70	58.1	CBM8
4884037_2	7291	965	GH53	Endo-1,4- $\beta$ -galactanase	2.92E-151	76.6	
5204286_1	6982	1044	GH128	Glycoside hydrolase	3.96E-103	66.8	

830359_1	6946	1312	GH1	$\beta$ -glucosidase	1.70E-140	68.5	
3175817_1	6832	1625	GH5_7	Endo-1,4- $\beta$ -mannanase	0	80.1	SS; MS
2132535_1	6435	1193	GH105	Glycoside hydrolase	4.62E-107	66.9	
1970615_2	5800	1048	GH7	Cellobiohydrolase 1	1.47E-134	76.5	MS

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<sup>1</sup>Complete information, including BLASTx analyses, Interpro and Gene Ontology designations, and mass spectrometry results are listed in supplemental Dataset S1.

<sup>2</sup>CAZy families identified by dbCAN (Yin et al 2012 NAR doi: [10.1093/nar/gks479](https://doi.org/10.1093/nar/gks479))

<sup>3</sup>e-value of the top BLASTx hit.

<sup>4</sup>Average sequence similarity to the top ten BLASTx hits.

<sup>5</sup>SS, SignalP determined secretion signal; MS, LC-MS/MS identified proteins; CBM, Carbohydrate Binding Module family.

**Table 4.** Glycoside hydrolases identified in decayed *Pinus contorta* by mass spectrometry<sup>1</sup>

Contig and frame	Reads	Length	CAZy	e-Value <sup>2</sup>	Coverage		# Pept	# PSMs	Unique Pept	Sequest HT score
			family		[%]					
4346763_2_5	1289	974	GH1	1.77E-123	12		3	5	3	13
4346763_4_2	134	980	GH1	5.00E-117	12		3	5	3	13
4346763_3_1	708	993	GH1	8.16E-121	9		2	3	2	8
4459897_1_1	260	884	GH10	1.63E-72	15		3	7	3	20
3889902_1_4	3745	1235	GH10	1.18E-172	7		2	5	2	17
2058669_1_6	2552	1145	GH10	8.35E-98	8		2	2	2	7
3960173_1_1	602	936	GH12	2.08E-142	6		2	4	2	12
3166842_1_5	1268	1146	GH17	1.92E-145	14		3	5	3	15
2491547_1_6	1188	1068	GH3	8.07E-91	13		2	5	2	5
3730185_1_5	1252	831	GH47	8.13E-166	8		2	3	2	8
6091156_1_3	25158	1281	GH6	2.70E-100	23		5	24	4	73
1553595_1_2	2108	926	GH6	5.22E-156	11		2	7	2	24
1384861_1_6	16216	1295	GH6	1.13E-102	8		2	4	1	8
1384861_2_1	4220	1303	GH6	2.48E-103	5		2	3	1	7
658106_4_6	1932	1273	GH7	1.72E-137	17		5	24	4	74
658106_7_2	1550	1024	GH7	1.50E-97	16		4	23	3	70
3240852_1_4	42911	1551	GH7	0	20		7	23	6	63
4852_1_6	697	764	GH7	3.56E-70	11		2	12	1	34
3223568_3_6	477	871	GH7	6.16E-115	10		2	9	1	26
3240852_2_6	1163	965	GH7	1.59E-104	16		3	9	3	26

1970615_2_6	5800	1048	GH7	2.18E-142	18	4	5	3	13
3589366_2_3	1295	1109	GH7	4.77E-168	14	3	3	2	5
3589366_1_6	13195	1464	GH7	0	6	2	2	1	4

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396 <sup>2</sup>BLASTp NR

397

Table 5. Redox enzymes potentially involved in lignocellulose degradation identified in decayed *Pinus contorta* by mass spectrometry.

Contig and frame	Read	Length	Description	CAZy family	e-Value <sup>2</sup>	Coverage [%]	Pept	PSMs	Unique Pept	Sequest HT score
4793784_3_2	4023	932	Manganese peroxidase	AA2	1.68E-159	7	2	3	1	8
4793784_13_2	2332	1097	Manganese peroxidase	AA2	0	8	2	3	1	9
2280897_3_6	3374	1119	Alcohol oxidase	AA3_3	0	26	8	23	8	66
2280897_9_1	1468	1170	Alcohol oxidase	AA3_3	0	20	6	11	5	34
798557_1_1	7736	1563	Alcohol oxidase	AA3_3	0	15	5	10	2	27
798557_5_2	3018	1095	Alcohol oxidase	AA3_3	0	17	4	8	1	20
798557_13_6	1908	1337	Alcohol oxidase	AA3_3	0	11	3	6	1	15
798557_17_6	2693	1051	Alcohol oxidase	AA3_3	2.12E-166	18	3	6	1	16
2280897_7_2	3087	1095	Alcohol oxidase	AA3_3	1.83E-177	11	2	5	1	15
2280897_11_6	1698	1169	Alcohol oxidase	AA3_3	0	9	3	3	2	9
798557_25_4	176	899	Alcohol oxidase	AA3_3	7.64E-157	13	2	5	0	12
536886_1_6	4574	1455	Alcohol oxidase	AA3_3	0	9	3	7	3	22
2766776_1_4	910	784	Benzoquinone reductase	AA6	7.36E-98	12	2	2	2	6
3005685_1_6	258	810	Benzoquinone reductase	AA6	6.80E-87	10	2	12	1	34
1341126_2_3	1595	1039	Benzoquinone reductase	AA6	7.50E-120	26	6	26	5	76
1507360_1_3	9562	1007	Benzoquinone reductase	AA6	1.28E-109	21	5	19	4	56
1507360_2_6	263	993	Benzoquinone reductase	AA6	2.95E-70	12	3	14	2	39
3719648_4_6	986	1106	Benzoquinone reductase	AA6	6.41E-111	27	8	43	7	123
1712785_3_2	1051	816	Benzoquinone reductase	AA6	8.34E-112	28	5	34	4	99
1712785_2_4	739	948	Benzoquinone reductase	AA6	5.49E-144	20	5	24	4	71

3711757_1_4	925	885	NADH-quinone OR	AA6	1.86E-106	7	2	10	1	27
3834150_1_3	5521	862	Benzoquinone reductase	AA6	9.20E-111	8	2	10	2	30
3719648_1_3	1059	828	NADH-quinone OR	AA6	5.06E-82	12	3	11	2	29
4644641_1_2	2872	842	Dyp-type peroxidase		3.56E-93	37	8	37	6	109
4644641_2_5	1109	863	Dyp-type peroxidase		4.33E-79	33	5	32	3	85
3735866_1_2	448	873	Dyp-type peroxidase		6.27E-45	20	3	17	2	51
3427787_2_2	1036	1107	Dyp-type peroxidase		1.96E-81	28	6	9	5	29

399 <sup>2</sup>BLASTp NR

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#### FIGURE LEGENDS

Figure 1. Distribution of 52,011 contigs. Hypotheticals includes conserved hypothetical, predicted proteins and uncharacterized proteins. Transporters include permeases and those assigned to the major facilitator superfamily (MFS)

Figure2. Number of contigs and reads assigned to AA families.

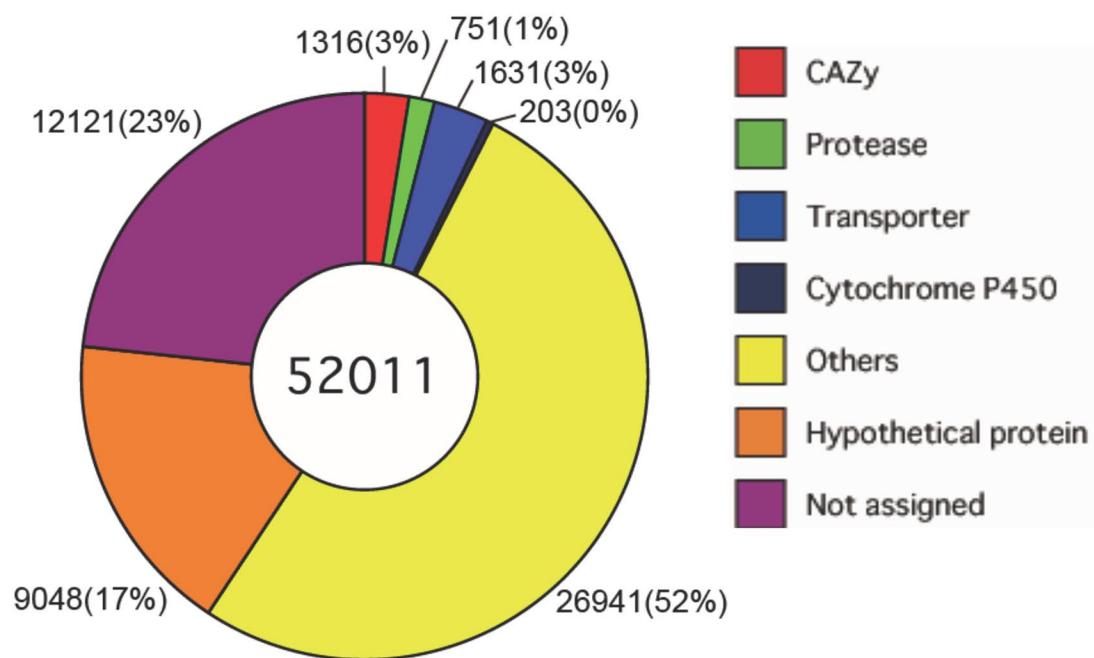
Figure 3. Number of contigs and reads assigned to glycoside hydrolase families known to include cellulases.

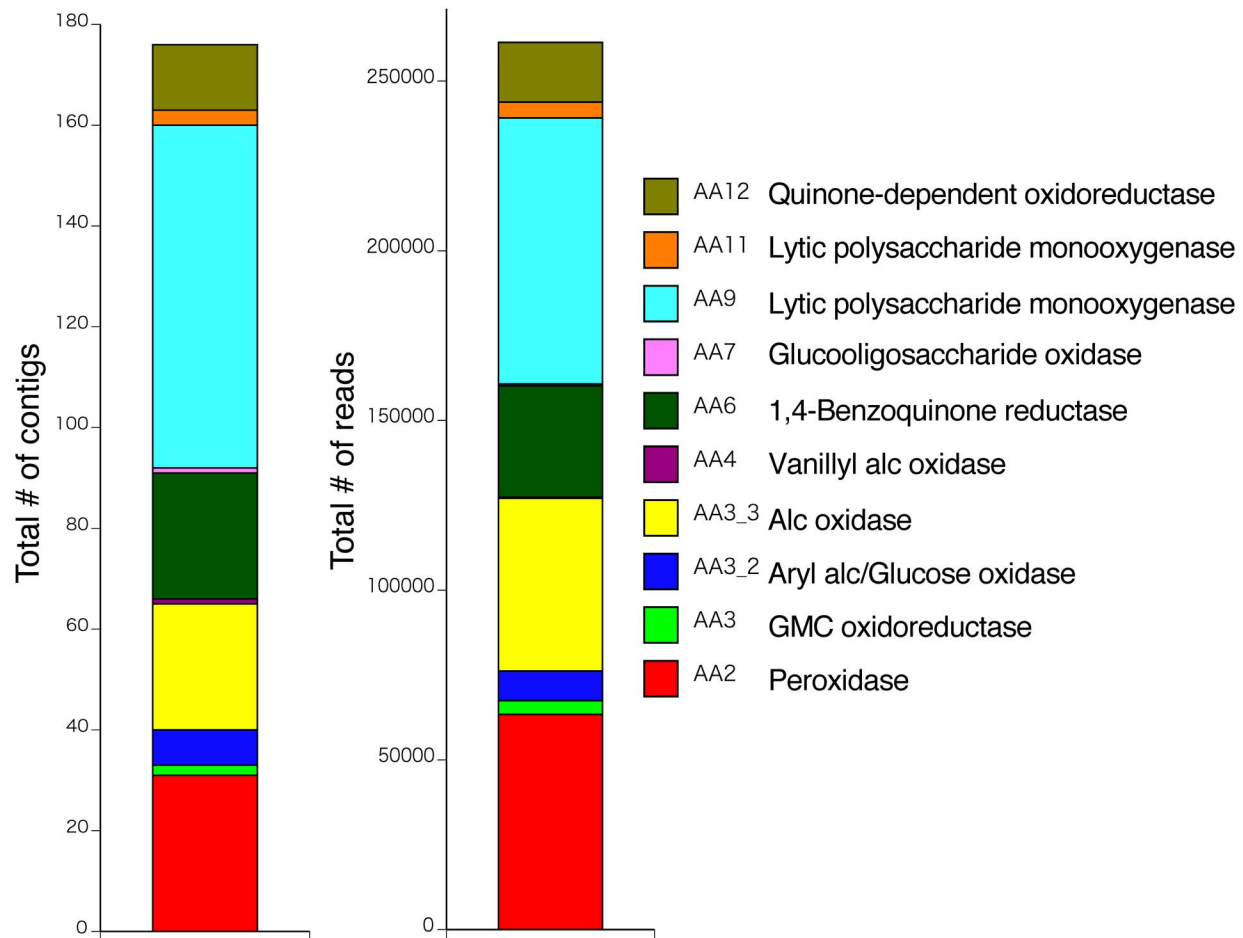
Figure 4. Number of contigs and reads assigned to glycoside hydrolase families known to include hemicellulases

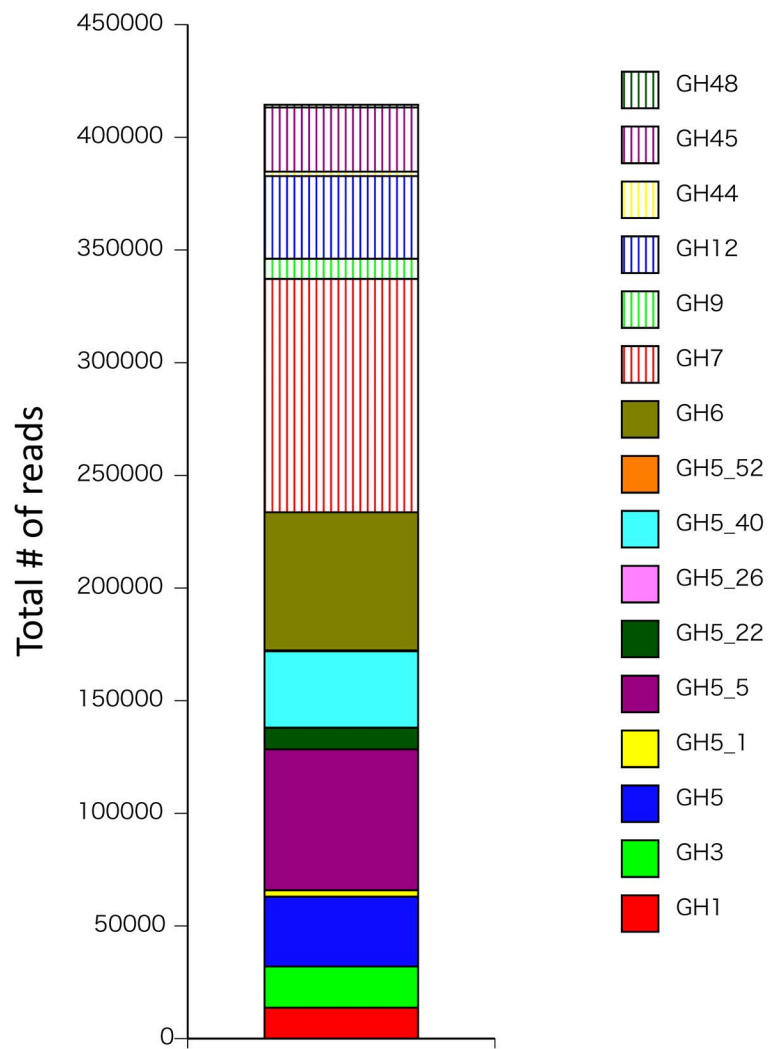
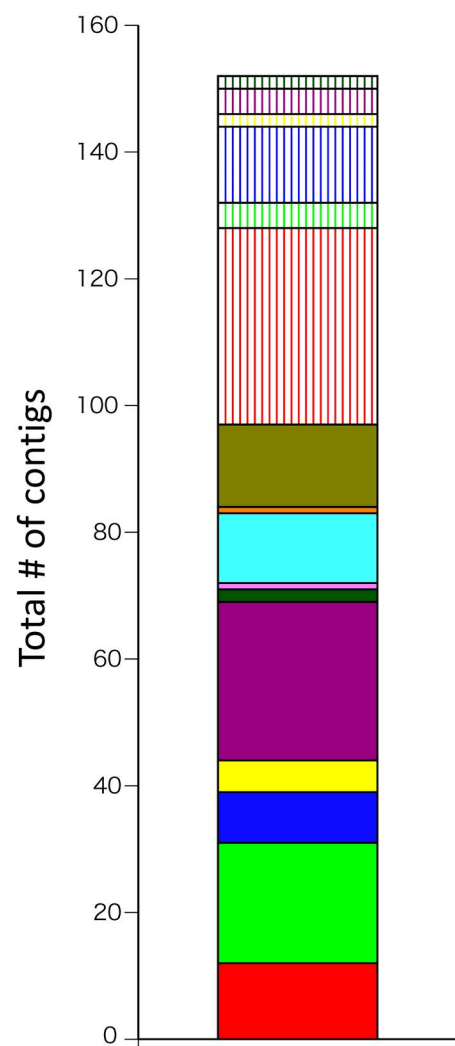


590 Figure 5. Distributions of top 30 BLASTp (NR database) hits among translations of all 52,011 contigs  
591 (left panel) and for 1,316 contigs (right panel) identified as CAzyme-encoding. The thirty species account  
592 for 43% and 54% of the total top hits for all proteins and the CAzyme subset, respectively. White and  
593 brown arrows indicate decay type, where known. Fill colors correspond to Basidiomycota (blue),  
594 Ascomycota (red) and various other eukaryotes or bacteria (green)

595  
596







- GH48
- GH45
- GH44
- GH12
- GH9
- GH7
- GH6
- GH5\_52
- GH5\_40
- GH5\_26
- GH5\_22
- GH5\_5
- GH5\_1
- GH5
- GH3
- GH1

